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PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF *VERBESINA ENCELIOIDES* (CAV.) BENTH. AND HOOK.

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ABSTRACT

Eight known compounds, including: taraxasterol acetate, ceryl alcohol (hexacosanol), β-amyrin, β-sitosterol and stigmasterol mixture, *p*-coumaric acid, β-sitosterol and stigmasterol-3-O-β-D-glucopyranoside mixture, quercetin-3-O-β-D-galactopyranoside and galegine (prenyl guanidine) were isolated from the petroleum ether and ethyl acetate fractions of hydroalcoholic extracts of the (branches of aerial parts and roots) and flowers of *Verbesina encelioides* (Cav.) Benth. and Hook. The structures of these compounds were elucidated using physicochemical characters and spectral methods: mass, UV, IR and NMR analyses. The total hydroalcoholic extracts possessed a promising cytotoxic activity against hepatocellular carcinoma using MTT assay. The total hydroalcoholic extract, petroleum ether and ethyl acetate fractions were active against Gram +ve bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram -ve bacteria (*Escherichia coli*) and fungi (*Aspergillus fumigatus* and *Candida albicans*) by the diffusion agar technique.

KEYWORDS

Verbesina encelioides, Phytochemical, Galegine, Cytotoxic and Antimicrobial.

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INTRODUCTION

Verbesina encelioides (Cav.) Benth. and Hook. is a plant belonging to family Compositae (Asteraceae)[1]. Previous phytochemical studies on genus *Verbesina* have shown the presence of sesquiterpene esters[2], sterols and triterpenes[3-5], saponins[6], flavonoids[7], alkaloids[8] and guanidine derivatives[8,9]. These compounds are characterized by their biological importance such as hypotensive[8],

hypoglycemic[10], cytotoxic[11], hypolipidemic[12], antibacterial[13,14], antifungal[14], antileishmanial[15]and nematicidal activity[16]. It is worthy to note that there is only one report on *Verbesina encelioides* (Cav.) Benth. and Hook. Cultivated in Egypt essential oil composition and the antimicrobial activity of these essential oils[13]. In the present study, organic extracts of *Verbesina encelioides* were phytochemically analysed and tested for some biological activities.

MATERIAL AND METHODS

Plant material

Verbesina encelioides was collected in the flowering stage in April 2013 from the experimental garden of Faculty of Pharmacy, Zagazig University, Egypt. The systematic identification of the plant was kindly verified by Dr. Hussein Abdel Baset, Professor of Botany, Faculty of Science, Zagazig University, Egypt. The plant was shade dried and ground by electric mill to moderately fine powder.

Apparatus and equipment

Melting points were carried out on melting point apparatus Gallen Kamp (United Kingdom). U.V. lamp for TLC visualization UVGL-55 (λ_{max} 254 and 365 nm). For U. V. spectral analysis, UV/VIS Jenway 6715 spectrophotometer was used. Infrared spectra were carried out by Jasco FT/IR 4100 spectrophotometer. Mass spectra were carried out by Mass Spectrometer Model: ISQ™LT and operate at 70 eV. ^{1}H-and ^{13}C-NMR spectra were recorded using Bruker at 400 and 100 MHz, respectively. TLC analyses were carried out on silica gel GF_{254} precoated plates with the developing solvent systems as listed in Table No.1. Silica gel (70-230 Mesh, Fluka) was used for column chromatography. Authentic reference materials (β-sitosterol, β-amyrin and β-sitosterol-3-O-β-D-glucopyranoside) were obtained from Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Extraction

The air-dried powdered branches of aerial parts and roots of *Verbesina encelioides* (Cav.) Benth. and Hook. (3.5 kg) was extracted by cold maceration with 70% ethanol (4×15L) till complete exhaustion. The combined extract was evaporated under reduced pressure at 50°C to give concentrated hydroalcoholic syrupy solution (900 ml) which was successively subjected to liquid-liquid fractionation with: petroleum ether (60-80°C)(4×2L), methylene chloride (4×2L) then with ethyl acetate (4×1L). The previous fractions were separately combined, washed with distilled water, dried over anhydrous sodium sulphate and concentrated under vacuum at 50°C to afford 71.3, 6.8 and 6.9 gm, respectively (greenish black residues).

The fresh flowers of *Verbesina encelioides* (Cav.) Benth. and Hook. (3.7 kg) was exhaustively extracted by cold maceration with 70% ethanol (4×10L). The combined extract was evaporated under reduced pressure at 50°C to give 600 ml of concentrated hydroalcoholic syrupy solution which was successively subjected to liquid-liquid fractionation with: petroleum ether (60-80°C) (4×2L), methylene chloride (3×3L) then with ethyl acetate (4×1L). The previous fractions were separately combined, washed with distilled water, dried over anhydrous sodium sulphate and concentrated under vacuum at 50°C to afford 16.5, 2.8 and 9.5 gm, respectively (greenish black residues).

Column chromatography of the petroleum ether fraction of the flowers of *Verbesina encelioides* (Cav.) Benth. and Hook. and isolation of four compounds

Due to the quite similarity of the TLC chromatograms of petroleum ether fractions of both (branches of aerial parts and roots) and flowers and that the petroleum ether fraction of the flowers contained lesser amount of chlorophyll and colouring matter about 10 gm of it was dissolved in 20 ml of methylene chloride and adsorbed on 20 gm silica gel and the dried mixed initial zone was placed on the top of a silica gel column (300 gm silica gel 70-230 Mesh, Fluka, 70×2 cm) packed by the wet method using petroleum ether. The elution was carried out starting with petroleum ether then the polarity increased gradually with methylene

chloride then methanol. Fractions (250ml each) were collected, concentrated and monitored by TLC. The similar fractions were pooled together and subjected to further purification processes. Fractions (70-79) eluted by petroleum ether: methylene chloride (6: 4) gave one major violet spot with R_f 0.73 (TLC, S_2) was subjected to repeated crystallization from methanol to give 43 mg of white needle crystals (compound 1). Fractions (94-113) eluted by petroleum ether: methylene chloride (2: 8) gave a colourless oily spot with R_f 0.26 (TLC, S_2) was subjected to repeated crystallization from methanol to give 21 mg of white crystals with m.p. 74-76°C (compound 2). Fractions (114-123) eluted by 100% methylene chloride gave two major spots with R_f 0.63 and 0.37 (TLC, S_3) was subjected to column chromatography to yield two sub fractions eluted by petroleum ether: methylene chloride (95: 5) and (9: 1). These subfractions were crystallized from methanol to yield 30 and 20 mg of compounds 3 and 4, respectively.

Column chromatography of the combined ethyl acetate fraction of *Verbesina encelioides* (Cav.) Benth. and Hook. and isolation of four compounds

Due to the quite similarity of the TLC chromatograms of ethyl acetate fractions of both (branches of aerial parts and roots) and flowers, both of them have been pooled together and about 10 gm of it was dissolved in 20 ml of methanol and adsorbed on 10 gm silica gel and the dried mixed initial zone was inserted on the top of a silica gel column (300 gm, 70×2 cm) packed by the wet method using petroleum ether. The elution was carried out starting with petroleum ether then the polarity increased gradually with methylene chloride then methanol. Fractions (250ml each) were collected, concentrated and monitored by TLC. The similar fractions were pooled together and subjected to further purification processes. Fractions (54-70) eluted by methylene chloride: methanol (92: 8) was column chromatographed to yield subfractions (96 -97) eluted by 0.5% methanol in methylene chloride. Those subfractions gave one purple spot with R_f 0.35 (TLC, S_5) which on crystallization from methanol gave 20 mg of compound 5. Fractions (76-82) eluted by methylene chloride: methanol (8: 2) was repeated crystallized from methanol to give 34 mg of white residue m.p. 288-294°C, R_f 0.65 (TLC, S_6). This substance was designated as compound 6. Fractions (85-90) eluted by methylene chloride: methanol (4: 6) gave two major spots with R_f 0.37 and 0.2 (TLC, S_6) was subjected to column chromatography. Subfractions (23-26) eluted by benzene: ethyl acetate (1: 1) revealed the presence of one major yellow spot with R_f 0.2 (TLC, S_6) which on crystallization from methanol gave 27 mg of compound 7. Subfractions (8-22) of this subcolumn was rechromatographed to yield subfractions (21-22) eluted by 4% methanol in methylene chloride and gave one major blue spot with R_f 0.37 (TLC, S_6) which on crystallization from methanol gave 37 mg of compound 8.

BIOLOGICAL EVALUATION

Cytotoxic activity

The total hydroalcoholic extracts, ethyl acetate fractions of both (branches of aerial parts and roots) and flowers of *Verbesina encelioides* (Cav.) Benth. and Hook. and the isolated compound 8 were tested for their in vitro cytotoxic activity against human hepatocarcinoma cell line (Hep G2) using the MTT Cell Viability Assay[17,18]. The tested extracts, fractions and isolated compound 8 were added into 96-well plates containing the 24 hours incubated tumor cell lines at different concentrations to achieve six concentrations for each of them. Six vehicle controls with media or 0.5 % DMSO were run for each 96-well plate as a control. After 24 hours, 12 mM MTT stock solution (5 mg of MTT in 1 ml of PBS) was added to each well including the untreated controls and incubated at 37°C and 5% CO_2 for 4 hours. Then, 50 µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 minutes. Then, the optical density was measured at 590 nm using a microplate ELISA reader (SunRise, Tecan, Inc, USA). Triplicate repeats were performed for each concentration and the average was calculated. The

percentage of viability was calculated and the percentage of cell viability was plotted against the tested sample concentrations and the IC_{50} was estimated from graphic plots of the dose response curve for each concentration using Graphpad Prism software (San Diego, CA, USA). The IC_{50} of tested samples were measured (μg/ml).

Antimicrobial activity

Diffusion agar technique[19] was used todetect the preliminary antibacterial and antifungal activity of different plant extracts including hydroalcoholic extract, petroleum ether and ethyl acetate fractions. The samples were dissolved in ethanol, methylene chloride and methanol, respectively at concentration 10 mg/ml. The nutrient agar (for bacteria) or Sabouraud Dextrose agar (for fungi) were seeded by Gram +ve bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram -ve bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungi (*Aspergillus fumigatus* and *Candida albicans*). Each cup was filled by about 100 μl of each extract (10 mg/ml). Ampicillin, Gentamicin and Amphotericin B were used as standards. The plates were incubated at 37°C over night (for bacteria) and at 30°C for 48 hours (for fungi). Mean zone of inhibition were measured (mm) and the data were expressed in the form of mean ± standard deviation.

RESULTS AND DISCUSSION

Identification of compounds

Compound 1: was isolated as white needle crystals (43 mg) with m.p. 246-247°C, R_f 0.73 (TLC, S_2). It showed positive Liebermann's and Salkowski tests indicating a triterpenoid and/or steroid skeleton.

The IR spectrum showed absorption frequencies: ν_{max} (KBr) cm^{-1}: 1727 and 1245 indicating ester group, 1640 indicating olefinic double bond, 2937, 2855 accompanied by 1450 and 1371represented the aliphatic methylene and methyl groups, respectively[20]. These data suggested the presence of a steroidal and/or triterpenoidal compound.

EI-MS exhibited a molecular ion peak at m/z 468 (M^+) which is compatible with the molecular formula $C_{32}H_{52}O_2$and a fragment ion peak at m/z408 (M^+-60) corresponds to the loss of acetate group. The base peak at m/z 189 and the fragments at m/z 399, 357, 249, 218, 205, 204 and 203 confirm strongly the steroidal and/or triterpenoidal skeleton[21].

From the previously mentioned characteristics beside the physicochemical characters of this compound and comparison with published data[21], the identity of compound 1 was confirmed to be taraxasterol acetate.

Compound 2: was isolated as white crystals (21 mg) with m.p. 74-76°C, R_f 0.26 (TLC, S_2). It gave negative Liebermann's test for sterols and/or triterpenes.

The IR spectrum showed a hydroxyl and C-OH stretching bands at 3318 and 1062 cm^{-1}, respectively. It also showed absorption bands at 2917, 2849 and 1466 cm^{-1} representing aliphatic stretching and bending peaks[20].

The MS fragmentation showed a fragment ion peak at *m/z* 364 (2 %) which corresponds to (M^+-H_2O). The base peak occurs at *m/z* 97 arises from the probable cleavage of a heptenyl moiety.

Strong peaks at m/z (% relative abundance): 336 (2.37), 308 (1.31), 265 (1.13), 251 (1.36), 237 (2), 223 (2.56), 209 (3.68), 195 (4.71), 181 (6.52), 167 (8.84), 153 (13), 139 (19), 125 (37), 111 (62), 97 (100), 83 (92), 69 (72), 67 (17), 57 (58), 55 (54), 43 (31) and 41 (18) with successive losses of 14 or 28 mass units were observed. All these observations indicated the presence of long chain alcohol[22].

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) exhibited a triplet at δ 0.88 (J = 7.2 Hz) probably for methyl group. The triplet at δ 3.64 (J = 6.8 Hz) for the CH_2OH group adjacent to methylene group. Protons of long chain CH_2 was countered at δ 1.25-1.57 and integrated for 48 protons.

The ^{13}C-NMR spectrum (100 MHz,$CDCl_3$) showed a methyl group carbon at δ 14.27 (assigned to C-26), a carbon linked to alcoholic oxygen at δ 63.27 (assigned to C-1), carbons adjacent to the alcoholic carbon at δ 32.98, 32.08 (assigned to C-2 and C-3, respectively), methylenic carbons neighboring to methyl group at δ 25.89, 22.84 (assigned to C-24 and C-25, respectively) and other methylenic carbons at δ 29.51 to 29.85 ppm.

The previous spectral analyses: IR, MS, ^{1}H and ^{13}C NMR suggested that this compound is a saturated, long chain aliphatic primary alcohol having 26 carbon atoms (ceryl alcohol or hexacosanol). This represents the first report for the isolation of this compound from genus *Verbesina*.

Compound 3: was isolated as white crystals (30 mg) with m.p. 197-198°C, R_f values 0.26 and 0.63 (TLC, S_2 and S_3, respectively). It showed positive Liebermann's and Salkowski tests indicating a triterpenoid and/or steroid skeleton.

The IR spectrum showed absorptions at 3412 and 1039 cm^{-1} indicating the presence of a hydroxyl group, absorptions at 1639, 994 and 879 cm^{-1} indicating the presence of double bond, absorption at 2940,2869 cm^{-1} accompanied by 1456 and 1382 cm^{-1} representing the aliphatic stretching and bending peaks[20]. These data suggested the presence of an unsaturated steroidal and/or triterpenoidal alcohol.

The mass spectrum showed molecular ion peak at m/z 426 suggesting a molecular formula $C_{30}H_{50}O$, a fragment ion peak at m/z 411 (M^+-15) corresponds to the loss of a methyl group and a fragment ion peak at m/z 408 (M^+-18) corresponds to the loss of water. The base peak at m/z 218 and the fragments at m/z 393, 272, 257, 231, 229, 207, 205, 204, 203 and 189 are characteristic for a monohydroxyl pentacyclic triterpene[21].

From the above mentioned spectral characteristics and direct comparison (co-tlc, m.p. and mass) with authentic β-amyrin and published data[21], compound 3 was confirmed to be β-amyrin.

Compound 4: was crystallized from methanol as white needles (20 mg) with m.p. 133-137°C, R_f 0.37 (TLC, S_3). It showed positive Liebermann's and Salkowski tests suggesting a steroid and/or triterpenoid skeleton.

The IR spectrum showed absorptions at 3429 and 1056 cm^{-1} representing the hydroxyl and C-OH stretching peaks, respectively. It also showed absorptions at 2936, 2867, 1641, 1459 and 1381 cm^{-1} representing the aliphatic stretching and bending peaks[20].

The mass spectrum of this compound showed a spectrum similar to that of sterols with two molecular ion peaks at *m/z* 414 and 412 which calculated for $C_{29}H_{50}O$ and $C_{29}H_{48}O$, respectively. Other fragments observed at m/z (% relative abundance): 399 (10), 397 (43), 396 (23), 394 (10), 369 (6), 329 (40), 327 (3), 303 (32), 301 (8), 273 (23), 271 (16), 255 (50) and 253 (20) are characteristic for Δ^5 - sitosterol and $\Delta^{5,\ 22}$ - stigmasterol skeletons[21].

From the previously mentioned spectral characteristics in addition to co-chromatography with authentic samples as well as comparison with the published spectral data[21], the identity of compound 4 was confirmed to be a mixture of β-sitosterol and stigmasterol.

Compound 5: was crystallized from methanol as yellowish brown needles (20 mg) with m.p. 211-213°C. It exhibited blue fluorescence in UV light at λ 365 nm indicating that it is a phenolic compound and purple spot with R_f values 0.35 and 0.9 (TLC, S_5 and S_6, respectively).

The IR spectrum indicated the presence of hydroxyl group stretching peak at 3389 cm^{-1}, aromatic carbon stretching peaks at 1599, 1510 and 1447 cm^{-1}. It also showed C=O peak of carboxylic acid at 1672 cm^{-1} and phenolic (C-OH) peak at 1213 cm^{-120}.

The mass spectrum of this compound showed a spectrum with molecular ion peak at *m/z* 164 (M^+, 45%) suggested for $C_9H_8O_3$ and significant fragments at *m/z* 147 (24%) corresponding to loss of hydroxyl group, 119 (43%) corresponding to loss of carboxylic group, 93 (11%) corresponding to the phenoxy group and 77 (10%) corresponding to the phenyl ring[22].

The ^{1}H-NMR spectrum (400 MHz, DMSO-d_6) showed two pairs of aromatic protons with doublet peaks at δ 7.49 and 6.78 with coupling constant 7.6 Hz, each integrated for two protons and assigned to H-2 and 6 and H-3 and 5, respectively. One pair of trans-olefinic protons neighboring to carboxylic group displayed at δ 7.46 and 6.26 with coupling constant 16 Hz, each integrated for one proton and assigned to H-7 and H-8, respectively.

The ^{13}C-NMR spectrum (100 MHz, DMSO-d_6) showed nine carbons at δ 143.92, 115.59 (for the two olefinic carbons neighboring to carboxylic group assigned to C-7 and C-8, respectively), δ 168.06 (for acid carbonyl group assigned to C-9), δ 159.54 (for C-4 which was substituted with hydroxyl group), δ 125.28 (for C-1), δ 130.01 (for C-2 and C-6 which were equivalent to each other) and δ 115.71 (for C-3 and C-5 which were also equivalent to each other).

From the above mentioned spectral data and comparison with the published data[23], it was concluded that compound 5 was 4-hydroxy cinnamic acid (*p*- coumaric acid). This represents the first report for the isolation of this compound from *Verbesina encelioides.*

Compound 6: was crystallized from methanol as white residue (34 mg) with m.p. 288-294°C, R_f 0.65 (TLC, S_6). It showed positive Liebermann's test indicating it's steroidal and/or triterpenoidal nature and positive Molisch's test indicating its glycosidic nature.

The IR spectrum indicated the presence of hydroxyl group and C-OH stretching peaks at 3423 and 1162 cm^{-1}, respectively. It indicated also the presence of aliphatic stretching and bending peaks at 2957, 2936, 1633, 1462 and 1378 cm^{-1}. The two peaks at 1075 and 1024 cm^{-1} indicated the presence of ether linkage[20].

The mass spectrum showed uncertain molecular ion peak. The two peaks at *m/z* 396 (71%) which calculated for $C_{29}H_{50}O$-18and 394 (16%) which calculated for $C_{29}H_{48}O$-18 indicated that this material may be a mixture of β-sitosterol and stigmasterol glycosides. The mass fragmentation pattern showed fragments at m/z 381 ($C_{29}H_{50}O$-18-CH_3), 379 ($C_{29}H_{48}O$-18-CH_3), 351, 301, 296, 288, 273, 271, 267, 253, 239, 231, 229, 213, 147 and 69 indicating a compound with Δ^5 - sitosterol and $\Delta^{5,22}$ -stigmasterol skeletons[21].

The ^{13}C NMR spectrum (100 MHz, DMSO-d_6) indicated 35 carbon signals for β-sitosterol-3-O-β-D-glucopyranoside, of which 29 were attributed to the aglycone moiety and 6 to the sugar moiety. The aglycone signals were at δ: 140.45 (C-5), 121.22 (C-6), 76.91 (C-3), 56.26 (C-14), 55.42 (C-17), 49.60 (C-9), 45.14 (C-24), 41.86 (C-13), 38.30 (C-4), 38.30 (C-12), 36.83 (C-1), 36.21 (C-10), 35.48 (C-20), 33.34 (C-22), 31.37 (C-7), 31.33 (C-8), 29.26 (C-2), 28.70 (C-25), 27.80 (C-16), 25.43 (C-23), 23.87 (C-15), 22.60 (C-28), 20.59 (C-11), 19.72 (C-26), 19.10 (C-19), 18.94 (C-27), 18.62 (C-21), 11.79 (C-29) and 11.68 (C-18). The carbon signals of the sugar moiety at δ: 100.78 (C-1'), 76.91 (C-3'), 76.76 (C-5'), 73.46 (C-2'), 70.10 (C-4') and 61.09 (C-6') were well consistent with those of glucose[24].

The spectrum also showed signals of stigmasterol-3-O-β-D-glucopyranoside, of which 29 signals were attributed to the aglycone moietyat δ: 140.45 (C-5), 138.04 (C-22), 128.83 (C-23), 121.22 (C-6), 76.91 (C-3), 56.26 (C-14), 56.17 (C-17), 50.59 (C-24), 49.60 (C-9), 41.86 (C-13), 41.74 (C-12), 38.30 (C-1), 36.83 (C-4), 36.21 (C-10), 35.48 (C-20), 33.34 (C-2), 31.42 (C-8), 31.37 (C-7), 31.33 (C-25), 29.26 (C-16), 24.87 (C-15), 23.87 (C-28), 22.60 (C-11), 19.72 (C-26), 19.10 (C-19), 18.85 (C-27), 18.62 (C-21), 11.79 (C-18) and 11.68 (C-29). The six signals of the sugar moiety at δ: 100.78 (C-1'), 76.76 (C-3'), 76.76 (C-5'), 73.46 (C-4'), 70.10 (C-2') and 61.09 (C-6').

From the above mentioned spectral characteristics, physicochemical characters and direct comparison (co-tlc) with authentic samples and published data[24,25]. It was concluded that compound 6 is a mixture of β-sitosterol and stigmasterol-3-O-β-D-glucopyranoside. This represents the first isolation of this mixture from *Verbesina encelioides.*

Compound 7: was isolated as yellow powder (27 mg) with m.p. 226-227°C. It exhibited blue fluorescence in UV light at λ 365 nm. It gave yellow spot with R_f value 0.2 (TLC, S_6) with NH_4OH and blue spot with $FeCl_3$indicating its falvonoidal nature. It gave positive Molisch's test indicating its glycosidic nature.

The IR spectrum showed hydroxyl group stretching peak at 3388 cm^{-1} and olefinic C-H stretching peaks of sugar moiety at 2966 and 2905 cm^{-1}. An aromatic carbon stretching peaks at 1557, 1504 and 1442 cm^{-1}. It showed also (C=O and C=C) peaks of ring A of

flavonoid structure at 1654 and 1605 cm^{-1}, respectively. The peaks at 1254, 1204, 1171, 1132, 1083, 1020 and 996 cm^{-1} (C-OH stretching) are indicative for the presence of several hydroxyl groups[20].

The mass spectrum showed uncertain molecular ion peak. The base peak at *m/z* 302 suggested for aglycone moiety with molecular formula $C_{15}H_{10}O_7$ and significant fragments at *m/z* 153 (13%) and 137 (19%) and 109 (9%) corresponding to $(A_1+H)^+$ and B_2^+ fragments resulted from pathway I and pathway II fragmentation pattern of O-falvonoidal glycosides, respectively[26].

The UV spectrum of compound 7 in methanol showed an absorbance bands at 360 nm (Band I) and 258 nm (Band II) indicating a flavonol skeleton. The UV spectrum revealed the occurrence of a bathochromic shift in band I ($\Delta\ \lambda$ = 48 nm) with increased intensity upon addition of sodium methoxide which indicated the presence of a free hydroxyl group at C-4'. A bathochromic shift in band II ($\Delta\ \lambda$ = 16 nm) after the addition of sodium acetate assigned for a free 7-OH group. A bathochromic shift ($\Delta\ \lambda$ = 78 nm) in band I in the UV spectrum of CH_3OH with $AlCl_3$ which was partially declined upon the addition of HCl by $\Delta\ \lambda$ = 38 nm indicated the presence of ortho-dihydroxyl groups as a substitution at ring B and a free hydroxyl group at C-3 or C-5. The presence of ortho-dihydroxyl groups in ring B (C-3' and C-4') was further confirmed by the bathochromic shift ($\Delta\ \lambda$ = 21 nm) of band I with sodium acetate/boric acid shift reagent[26].

^{1}H-NMR (400 MHz, DMSO-d_6) spectrum showed the protons of ring B as a singlet at δ 7.53 assigned to H-2' and a doublet at δ 6.80 (J = 8.4 Hz) assigned to H-5' due to ortho coupling with H-6' which appeared at δ 7.66 (J = 8.4 Hz). The two singlets at δ 6.20 and 6.41 each integrated for one proton were corresponding to H-6 and H-8, respectively. The doublet at δ 5.37 (J =7.6 Hz) assigned for the anomeric proton H-1" of a β-galactopyranoside moiety linked to quercetinaglycone at C-3.

^{13}C NMR (100 MHz, DMSO-d_6) spectrum showed signals for five hydroxylated carbons at δ 164.11 (C-7), 161.20 (C-5), 148.44 (C-4'), 144.81 (C-3') and 133.45 (C-3) and C=O carbon at δ 177.47 in addition to the other characteristic chemical shift for quercetin 3-O-β-D-galactopyranoside carbons at δ 156.27 (C-2), 156.27 (C-9), 121.97 (C-6'), 121.06 (C-1'), 115.91 (C- 5'), 115.16 (C-2'), 103.89 (C-10), 101.76 (C-1"), 98.64 (C-6), 93.47 (C-8), 75.82 (C-5"), 73.16 (C-3"), 71.17 (C-2"), 67.89 (C-4") and 60.10 (C-6").

From the previously mentioned spectral data and comparison with the published data[27], it was concluded that compound 7 was quercetin 3-O-β-D-galactopyranoside which was isolated before from *Verbesina encelioides*[7].

Compound 8: was isolated as buff crystals (37 mg) with m.p. 62-63°C. It exhibited blue fluorescence in UV light at λ 365 nm. It gave blue and orange spots with R_f value 0.37 (TLC, S_6) with anisaldehyde/ sulphuric acid and Dragendorff's as spraying agents, respectively.

The IR spectrum showed amino groups stretching peaks at 3404, 3271 (for NH_2) and 3202 (for NH). It also indicated the presence of H-C=C, C=N and C=C stretching peaks at 3022, 1670 and 1624 cm^{-1}, respectively which suggested the presence of alkenyl guanidine derivative[20].

The mass spectrum of this compound showed a spectrum with molecular ion peak at *m/z* 127 (M^+, 14%) suggested for $C_6H_{13}N_3$ and significant fragments at *m/z* 112 (100 %) corresponding to loss of methyl group, 84 (53 %) corresponding to loss of propyl group, 70 (42 %) corresponding to loss of butyl group, 57 (12 %) corresponding to butyl radical and 43 (33 %) corresponding to propyl radical.

^{1}H-NMR (400 MHz, DMSO-d_6) spectral data showed two olefinic methyl signals at δ 1.64 and 1.71 together with one olefinic proton as a triplet at δ 5.17 coupled (J = 5.2 Hz) to an adjacent doublet methylene group at δ 3.70 indicated for dimethylallyl moiety attached to an electronegative atom or group. The downfield signal at δ 7.44 was assigned to NH proton linked to alkenyl carbon. The broad singlet signal at δ 7.01, integrated for

three protons and was assigned to the NH_2 and NH signals.

^{13}C-NMR (100 MHz, DMSO-d_6) spectral data showed six carbons at δ 25.25, 17.77 (for the two methyl groups of the dimethylallyl moiety assigned to C-4 and C-5, respectively), δ 118.97, 136.35 (for alkenyl carbons assigned to C-2 and C-3, respectively), δ 40 (for C-1 which was attached to guanidine group and hidden by DMSO peak) and δ 156.50 (for C=NH of the guanidine moiety).

From the above mentioned spectral data and comparison with the published data[8,9], it was concluded that compound 8 was N-(3-methyl-2-butenyl)guanidine (galegine) which was isolated before from *Verbesina encelioides*[9].

Cytotoxic activity

As shown in Table No.2, the total hydroalcoholic extracts of *Verbesina encelioides* (Cav.) Benth. and Hook. Possessed a promising cytotoxic activity against hepatocellular carcinoma while the ethyl acetate fractions and isolated compound 8 (galegine) were ineffective in treatment of hepatic cancer.

Antimicrobial activity

As shown in Table No.3, The total hydroalcoholic extract and ethyl acetate fraction showed high antimicrobial activity where the petroleum ether fraction showed moderate activity against Gram +ve bacteria, *Escherichia coli*and fungi while they had no activity against *Pseudomonas aeruginosa*. The activities of the extracts were less than those of reference drugs except in case of activity of total hydroalcoholic extract against *Candida albicans* which was higher than amphotericin B reference antifungal drug.

BIOLOGICAL STUDIES

Table No.1: Chromatographic solvent systems for TLC

S.No	No	Solvent system	Ratio
1	S_1	Petroleum ether: methylene chloride	7: 3
2	S_2	Petroleum ether: methylene chloride	2: 8
3	S_3	Methylene chloride	Pure
4	S_4	Methylene chloride: methanol	95: 5
5	S_5	Methylene chloride: methanol	92: 8
6	S_6	Benzene: ethyl acetate: formic acid: water	3: 5: 1.6: 0.4

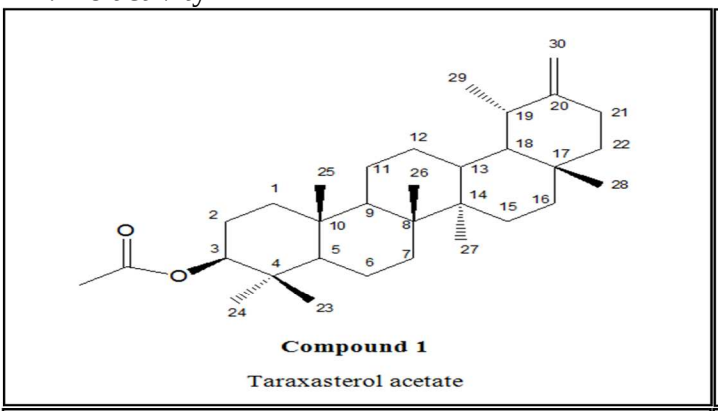
Table No.2: IC_{50} values of different plant extracts and isolated compound 8

S.No	Tested sample	IC_{50} against Hep G2 cell line
1	Total hydroalcoholic extract of branches of aerial parts and roots	5.5 µg/ml
2	Total hydroalcoholic extract of flowers	17.7 µg/ml
3	Ethyl acetate fraction of branches of aerial parts and roots	43.6 µg/ml
4	Ethyl acetate fraction of flowers	21.6 µg/ml
5	Isolated compound 8	10.9 µg/ml

Table No.3: Mean zone of inhibition in mm ± Standard deviation beyond well diameter (6 mm) using (10 mg/ml) concentration of tested plant extracts

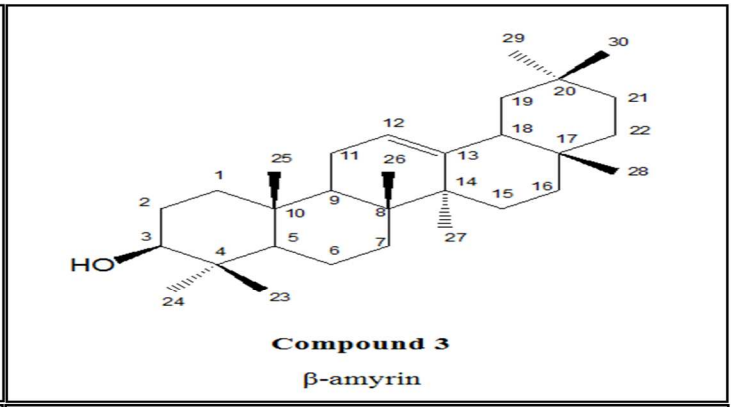
Tested extract / Tested microorganism	Total hydroalcoholic extract	Petroleum ether fraction	Ethyl acetate fraction	Standard
Fungi				Amphotericin B
Aspergillus fumigatus	21.3 ± 1.2	19.3 ± 0.63	22.9 ± 0.58	23.7± 0.10
Candida albicans	22.3± 0.58	20.1± 0.58	20.1 ± 1.2	21.9± 0.22
Gram +ve bacteria				Ampicillin
Staphylococcus aureus	19.8 ± 0.63	16.2 ± 1.2	23.2 ± 0.63	27.4± 0.18
Bacillis subtilis	22.3 ± 1.5	18.6 ± 0.72	20.9 ± 0.62	32.4± 0.10
Gram -ve Bacteria				Gentamicin
Pseudomonas aeruginosa	NA	NA	NA	17.3 ± 0.15
Escherichia coli	19.8± 1.2	15.6± 0.63	20.1 ± 1.5	22.3± 0.18

NA: No activity



Compound 1

Taraxasterol acetate



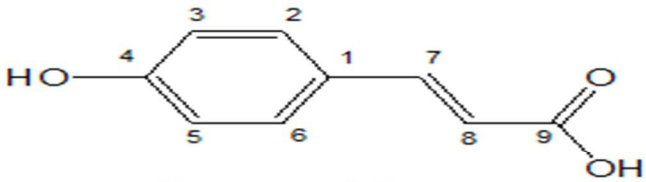
Compound 3

β-amyrin

$CH_3\text{-}CH_2\text{-}CH_2\text{-}(CH_2)_{20}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}OH$

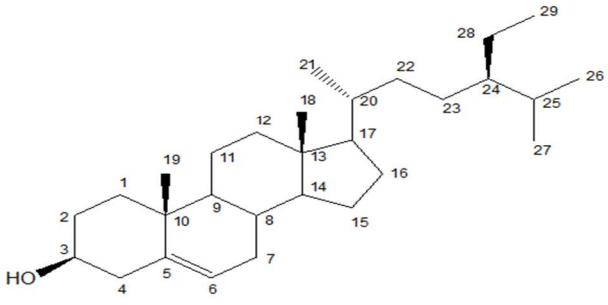
Compound 2

Hexacosanol (Ceryl alcohol)



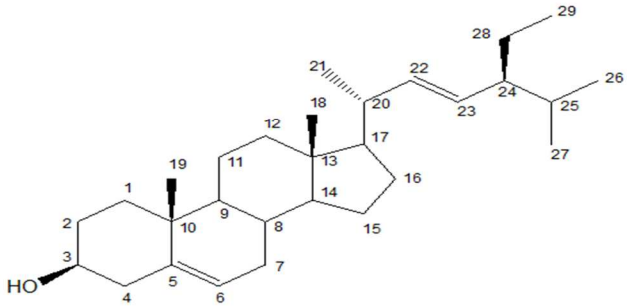
Compound 5

p-coumaric acid



Compound 4a

β-sitosterol



Compound 4b

Stigmasterol

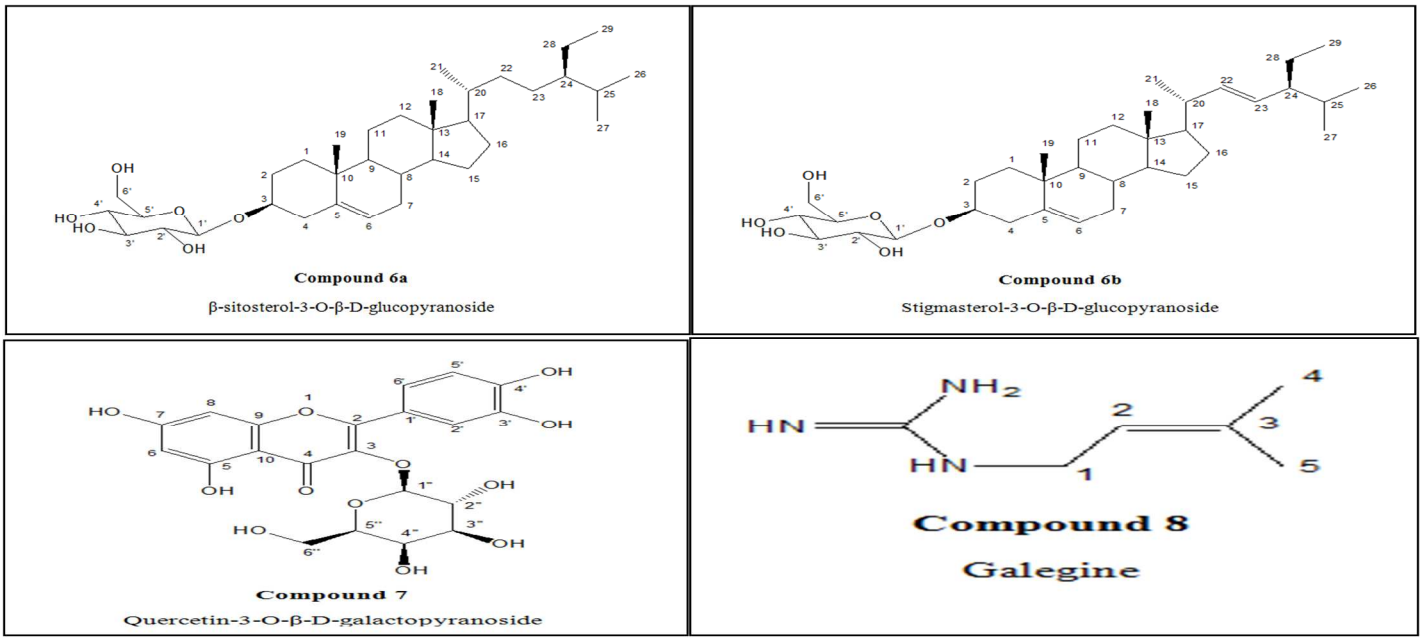
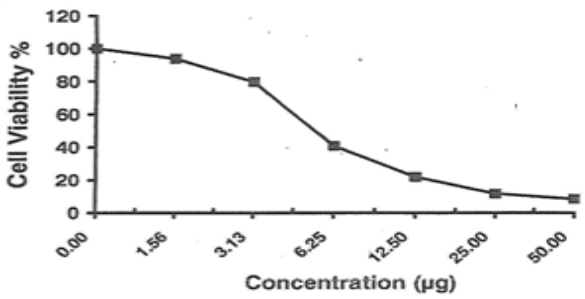
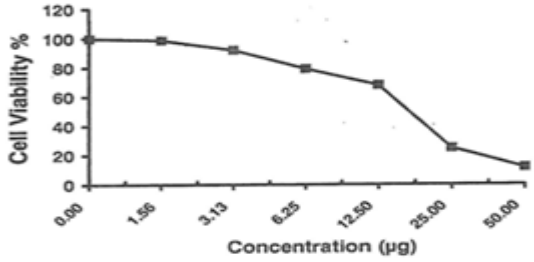


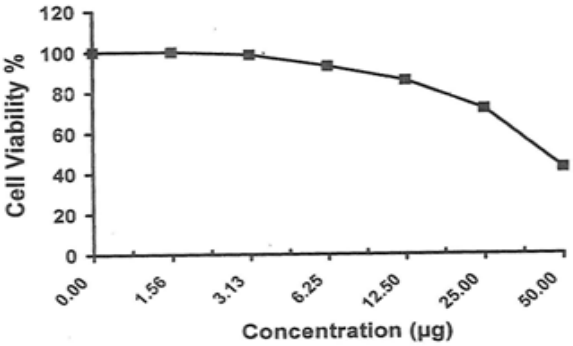
Figure No.1: The chemical structures of the isolated compounds from *Verbesina encelioides* (Cav.) Benth. and Hook.



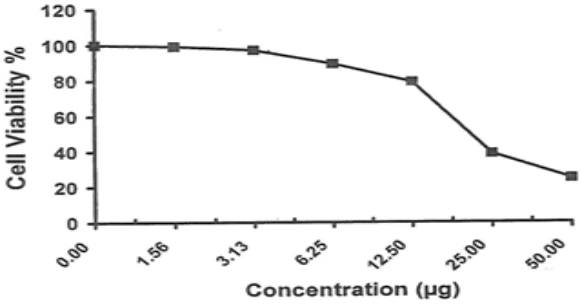
Total hydroalcoholic extract of branches of aerial parts and roots against Hep G2 cells: IC_{50}= 5.5 µg/ml



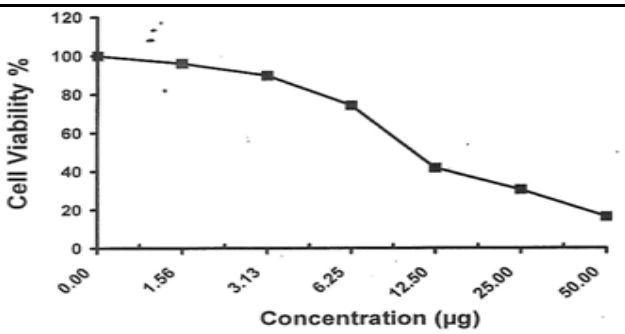
Total hydroalcoholic extract of flowers against Hep G2 cells: IC_{50} = 17.7µg/ml



Ethyl acetate fraction of branches of aerial parts and roots against Hep G2 cells: IC_{50} = 43.6 µg/ml



Ethyl acetate fraction of flowers against Hep G2 Cells: IC_{50} = 21.6 µg/ml



Isolated compound 8 (Galegine) against Hep G2 cells:

IC_{50} = 10.9 µg/ml

Figure No.2: Cytotoxic activity of different plant extracts and isolated compound 8 against Hep G2 cell lines

CONCLUSION

In the present study phytochemical and biological investigation of *Verbesina encelioides* (Cav.) Benth. and Hook. cultivated in Egypt was carried out. By phytochemical study the isolation of taraxasterol acetate, ceryl alcohol, β-amyrin, β-sitosterol and stigmasterol mixture from the petroleum ether fraction and *p*-coumaric acid, β-sitosterol and stigmasterol-3-O-β-D-glucopyranoside mixture, quercetin-3-O-β-D-galactopyranoside and galegine from the ethyl acetate fraction was achieved. This finding represents the first isolation of these compounds from *Verbesina encelioides* (Cav.) Benth. and Hook. cultivated in Egypt. Cytotoxic and antimicrobial studies were carried out on various extracts and fractions of the plant indicating that the total hydroalcoholic extracts of both (branches of aerial parts and roots) and flowers had a promising cytotoxic activity against hepatocellular carcinoma and that the total hydroalcoholic extract, petroleum ether and ethyl acetate fractions possessed a significant antibacterial and antifungal effect.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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